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► **To cite this version:**

Tom Van J M Dooren, Amanda Bortolini Silveira, Elodie E. Gilbault, José M Jiménez-Gómez, Antoine Martin, et al.. Mild drought induces phenotypic and DNA methylation plasticity but no 1 transgenerational effects in *Arabidopsis* 2 3. 2019. hal-02398707

HAL Id: hal-02398707

<https://hal.science/hal-02398707>

Preprint submitted on 7 Dec 2019

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1 **Mild drought induces phenotypic and DNA methylation plasticity but no**
2 **transgenerational effects in Arabidopsis**

3

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23 Total word count main body: 6412

24 Summary: 195 words

25 Introduction: 991 words

26 Materials and Methods: 1941 words

27 Results: 2369 words

28 Discussion: 1014 words

29

30 Main text: 4 figures (color) and 3 tables

31 Supplementary material: 4 figures (color) and 8 tables

32

33

34 **Summary**

35 Whether environmentally induced changes in phenotypes can be heritable is a topic with
36 revived interest, in part because of observations in plants that heritable trait variation can
37 occur without DNA sequence mutations. This other system of inheritance, called
38 transgenerational epigenetics, typically involves differences in DNA methylation that are
39 stable across multiple generations. However, it remains unknown if such a system responds to
40 environmental changes and if it could therefore provide a rapid way for plants to generate
41 adaptive heritable phenotypic variation. Here, we used a well-controlled phenotyping
42 platform and whole-genome bisulfite sequencing to investigate potential heritable effects of
43 mild drought applied over two successive generations in *Arabidopsis thaliana*. Plastic
44 phenotypic responses were observed in plants exposed to drought. After an intervening
45 generation without stress, descendants of stressed and non-stressed plants were phenotypically
46 indistinguishable, except for very few trait-based parental effects, and irrespective of whether
47 they were grown in control conditions or under water deficit. Moreover, while mild drought
48 induced changes to the DNA methylome of exposed plants, DNA methylation variants were
49 not inherited. These findings add to the growing body of evidence indicating that
50 transgenerational epigenetics is not a common response of plants to environmental changes.

51

52 **Keywords:** Arabidopsis, drought, plasticity, methylation, transgenerational effects,
53 epigenetics

54

55 **Introduction**

56 It is now well established that DNA mutations are not the only source of heritable phenotypic
57 variation in plants. An additional system of inheritance, often referred to as transgenerational
58 epigenetics, typically involves stable differences of DNA methylation at or near transposable
59 element (TE) sequences that are adjacent to genes (Quadrana & Colot, 2016). In the reference
60 plant *Arabidopsis*, most TE sequences are methylated at all cytosines, with methylation levels
61 generally highest at CG sites (>80%), intermediate at CHG sites (40-60%) and lowest at CHH
62 sites (<20%) (Cokus *et al.*, 2008; Lister *et al.*, 2008). TE sequences are methylated as a result
63 of the combined activity of multiple DNA methyltransferases (Law & Jacobsen, 2010; Stroud
64 *et al.*, 2013; Stroud *et al.*, 2014) and can also be actively demethylated by DNA glycosylases,
65 which excise methylated cytosines from DNA (Law & Jacobsen, 2010). Demethylation is
66 most pronounced in the central cell and leads after fertilization to a global hypomethylation of
67 TE sequences in the endosperm, specifically on the maternally-derived chromosomes (Satyaki

68 & Gehring, 2017). In contrast, because methylation dynamics is restricted to CHG and
69 especially CHH sites (Bouyer *et al.*, 2017; Kawakatsu *et al.*, 2017; Lin *et al.*, 2017), TE
70 sequences remain highly methylated in the female and male germlines as well as the embryo.
71 In turn, this limited reprogramming of DNA methylation patterns between generations implies
72 a considerable potential genome-wide for transgenerational epiallelic variation following
73 accidental loss of DNA methylation. However, because the *de novo* DNA methylation
74 machinery targets distinct TE sequences with varying efficiency (Teixeira *et al.*, 2009;
75 Zemach *et al.*, 2013), this potential is in fact not uniformly distributed among TE-containing
76 alleles. Thus, while experimentally induced epiallelic variation can persist for at least 8
77 generations and presumably many more at some TE-containing loci, it is fully erased within
78 one or a few generations at others (Johannes *et al.*, 2009; Teixeira *et al.*, 2009; Colome-
79 Tatche *et al.*, 2012).

80 Being sessile organisms, plants are often exposed to environmental stresses, which can have a
81 profound impact on the growth and development of not only the exposed individuals but also
82 their offspring. These parental (typically maternal) effects are well documented (Blödner *et al.*,
83 2007; Galloway & Etterson, 2007; Donohue, 2009; Herman & Sultan, 2011; Crisp *et al.*,
84 2016; Van Dooren *et al.*, 2016). In contrast, few experimental studies have been conducted to
85 determine if a phenotypic memory of environmental stress can persist over multiple
86 successive generations. In one early study, genetically identical Arabidopsis lines grown
87 under mild heat during the reproductive phase (from bolting onward) over two generations
88 and then grown under normal conditions for one more generation produced progeny with an
89 ameliorated response to heat treatment compared to control progeny derived from non-treated
90 lines (Whittle *et al.*, 2009). However, as heat was applied during reproductive growth, it
91 affected the gametes and the developing seeds produced by the treated plants (Whittle *et al.*,
92 2009). Therefore, parental effects could still be responsible for the ameliorated response to
93 heat seen in the progeny of these individuals (Blödner *et al.*, 2007; Pecinka & Mittelsten
94 Scheid, 2012). Consistent with this possibility, another study indicated that when heat stress
95 was applied during vegetative growth only, phenotypic effects did not persist for more than
96 one generation (Suter & Widmer, 2013b; Suter & Widmer, 2013a). Similarly, when
97 Arabidopsis plants were infected with pathogens, increased resistance was reported in the
98 immediate progeny as well as in the second generation, but only when infections were carried
99 out until the reproductive phase (Boyko *et al.*, 2010; Luna *et al.*, 2012; Slaughter *et al.*, 2012).

100 Salt stress memory across generations was also investigated and findings all point to an
101 absence of *bona fide* transgenerational effects (Boyko *et al.*, 2010; Suter & Widmer, 2013b;

102 Suter & Widmer, 2013a; Groot *et al.*, 2016; Wibowo *et al.*, 2016). Thus, the moment when
103 stress is experienced in the plant life history matters for the strength of the effect, its
104 transmission across generations and probably the causation of the effects. Finally, in the few
105 cases where this was looked at, DNA methylation changes were observed in response to stress
106 and some of these changes were transmitted, but again transmission was limited to the
107 immediate progeny only (Secco *et al.*, 2015; Wibowo *et al.*, 2016; Ganguly *et al.*, 2017).
108 Here, we set out to determine if prolonged water deficit, a common stress that plants face in
109 natural settings, could lead to new or altered transgenerational effects. Our experimental
110 design and analysis combine several distinctive key aspects compared to previous studies on
111 responses to stress. First, we tested four accessions (Shahdara, Bur-0, Tsu-0 and Cvi-0) in
112 addition to the reference accession Col-0, in order to assess how stress response, possibly in
113 relation to distinct TE landscapes, could influence phenotypic patterns. Second, we used a
114 well-controlled multigenerational experimental design where the magnitude and type of
115 environmental stress was replicated across generations for all five accessions. This design
116 enabled us to readily distinguish parental from transgenerational effects and to investigate
117 interactions of maternal effects and plasticity. Third, we used the robotic platform
118 Phenoscope, which ensures uniform conditions during vegetative growth and enables
119 continuous as well as precise phenotype tracking. Fourth, we analyzed the development of
120 phenotypes over time, and estimate magnitudes of trait-based maternal effects for five traits in
121 combination with plasticity. Fifth, we carried out an in-depth assessment of the likelihood of
122 transgenerational epigenetics at the DNA methylation level in the reference accession Col-0.
123 Our results indicate that mild drought induces phenotypic plasticity in each of the five
124 accessions, but does not lead to any significant change in terms of heritable effects. In
125 addition, we obtained methylome data at single cytosine resolution for Col-0, which again
126 indicate that mild drought induces intragenerational DNA methylation changes only, which
127 are restricted to CHH sites and affect TE sequences predominantly. Taken together, our
128 findings add to the growing body of evidence indicating that plants do not commonly generate
129 transgenerational effects in response to changes in the environment.

130

131 **Material and methods**

132 **Plant material and growth conditions**

133 To investigate interactions between genotype and environment (G×E) in the response to mild
134 drought (Bouchabke *et al.*, 2008), we considered the five accessions Col-0 (Col; Versailles
135 stock # 186AV), Shahdara (Sha; 236AV), Bur-0 (Bur; 172AV), Tsu-0 (Tsu; 91AV) and Cvi-0

136 (Cvi; 166AV), which were obtained from the Versailles stock center
137 (<http://publiclines.versailles.inra.fr/>). The four non-reference accessions were chosen because
138 they show similar flowering time to Col-0 but extensive nucleotide as well as DNA
139 methylation divergence among themselves and with Col-0 (Consortium, 2016; Kawakatsu *et*
140 *al.*, 2016a). Isogenic lines for each accession were grown under well-watered control and mild
141 drought stress conditions for three generations (Fig. 1), using the robotic platform Phenoscope
142 (<https://phenoscope.versailles.inra.fr/>), which ensures uniform conditions during vegetative
143 growth and enables precise phenotype tracking (Tisne *et al.*, 2013). At the first generation
144 (G1), 12 individuals (descending from the same mother plant) per accession and per treatment
145 were grown and half were used to establish 6 independent founder lines, which were then
146 maintained throughout the experiment by single seed descent propagation. In the following
147 generations, six replicates per accession, treatment and trajectory (life history) were grown,
148 with the exception of the third generation (G3) where only four replicates were grown due to
149 space limitations on the phenotyping robot (Fig. 1). Growth conditions (control and mild
150 drought stress) in each generation were as described in detail elsewhere (Tisne *et al.*, 2013).
151 Briefly, seeds were stratified for 4 days in the dark at 4°C and germinated for 8 days on peat
152 moss plugs before the plugs were transferred to the robot. Individual plants were then
153 cultivated on the Phenoscope for another 21 days under short days in order to minimize
154 developmental differences between accessions and delay flowering transition. The first week,
155 during germination, soil was saturated with water. During the first 7 days of growth on the
156 Phenoscope (Day 9 to 15 after sowing), soil water content gradually decreased through
157 controlled watering until it reached either 60% (control) or 30% (stress), which was then
158 strictly maintained at this level for the remaining 2 weeks (until Day 29 after sowing). At the
159 end of the Phenoscope experiment, plants were moved to a standard growth chamber with
160 optimal watering and long-day conditions to allow for flowering and seed production. This
161 strategy ensured that gametes or seeds were not themselves exposed to mild drought.
162 For the subsequent generation, collected seeds were sieved so as to avoid sowing seeds that
163 were in the top 10% size or the bottom 10% of each line's seed size distribution. Seed size
164 range varied between accessions (with Cvi and Bur having bigger seeds than Col and Sha
165 especially), but not between lines within accessions. A final phenotyping experiment (P4; Fig.
166 1) was conducted on lines from two selected trajectories (S1S2C3 versus C1C2C3).

167

168 **Phenotyping**

169 At every generation, zenithal rosette images of each individual plant were taken daily and
170 segmented as described previously (Tisne *et al.*, 2013) to extract projected rosette area (PRA;
171 a good proxy for rosette biomass at these development stages), rosette radius (the radius of the
172 circle encompassing the rosette), compactness (the ratio between PRA and the rosette circle
173 area) as well as the red, green and blue components of the segmented rosette image. We
174 report our phenotypic analysis of the last generation of plants grown on the Phenoscope (P4).

175

176 **Size and relative growth analysis**

177 Plant cohorts might contain different groups with different properties and responses to
178 treatments, while group membership might be unknown for each individual. To investigate
179 such large initial heterogeneity among the plants selected for growth on the robot, initial PRA
180 distributions on Day 9 after sowing (essentially the summed cotyledon areas) were inspected
181 by finite mixture analysis using the FlexMix library (Grun & Leisch, 2007). Gaussian models
182 for initial $\log(\text{PRA})$ with different numbers of component distributions (1 to 3) and different
183 fixed treatment effects (stress/control in P4, stress/control in G1 and G2, i.e. G1/G2) for each
184 component were compared using three information criteria (AIC, BIC and ICL). The model
185 with the lowest values of the information criteria was chosen each time, with preference given
186 to the ranking of ICL in case of discordance. Significant fixed effects are reported (z -test) for
187 preferred models, where these occur.

188 Initial and final values of $\log(\text{PRA})$ were further studied in detail using linear mixed models
189 (Pinheiro & Bates, 2000). The maximal models fitted contained random line effects with
190 different variances in control and stress groups in G1/G2. Models with more involved random
191 effect specifications did not converge. Fixed effects were the exposure to stress in G1/G2,
192 stress treatment in P4, pot order effects on the robot and interactions of these variables. The
193 maximal model contained heterogeneous error variances, different for each control/stress
194 combination in G1/G2 and P4. Model comparisons and simplifications were carried out using
195 likelihood ratio tests LRT (using a REML fit for random effects, ML for fixed). Non-
196 significant effects were removed one by one. Simplifications were first attempted in the
197 random effects, then in the error variances and after that in the fixed effects, starting with the
198 highest order interactions. Tests and estimates for random effects and error variances are
199 reported for a REML model containing all fixed effects. P-values for fixed effects are reported
200 with respect to the minimum adequate model (MAM) selected, i.e., the first model
201 encountered that only contains significant effects.

202 We also analyzed relative growth rates of PRA to understand the gradual response to
203 environmental conditions. We first inspected the per-day relative increase of log(PRA) using
204 generalized additive mixed models (gamms; (Wood, 2017) and subsequently fitted mixed
205 linear models (Pinheiro & Bates, 2000) to growth rates in age intervals that appeared linear.
206 Per separate treatment combination and accession, a gamm was fitted with smooths for day
207 (age) and pot order effects (pot number). The gamm's assume random variation between
208 individuals and exponentially decaying correlations between observations on the same
209 individual. The mixed linear models fitted to restricted age intervals contained the same
210 variables as the models for final size above, with the addition of random variation between
211 individuals within lines, fixed age (day) effects and interactions of age with the other fixed
212 effects.

213

214 **Phenotypic maternal trait-based effects**

215 Our analysis of PRA is detailed and accounts for effects of ancestral environments in G1/G2,
216 a potentially transmitted maternal environmental effect, but does not include trait-based
217 maternal effects (Kirkpatrick & Lande, 1989). To investigate these effects and whether
218 ancestral environments (i.e. memory) affect their strength and transmission, we analyzed all
219 traits recovered from digital images in a manner that could be applied to all traits equally. We
220 restricted the analysis to the set of traits measured in both G3 and P4 and with correlations
221 between them in P4 below 0.9 (for instance the image green component mean was too
222 correlated to the red component mean to be included as well). Per accession, we thus fitted
223 linear mixed effects models to the log-transformed trait values after day 23. This part of the
224 age trajectory of these traits was always approximately linear. For all traits, we tested whether
225 trait-based parental effects were present and differed between treatments in G1/G2 (ancestral
226 environment) and P4 (plasticity). We used maternal trait values on day 29 of G3 as
227 explanatory variables to model the trait-based effects. We removed data on a few plants with
228 outlying patterns for the increase in log(PRA) before analysis: Observations with a Cook's
229 distance value (in a simple regression on age) that was larger than one over the number of
230 observations were removed. We fitted maximal mixed models to the data per accession and
231 per trait with random effects of line and individual and heterogeneous error variances that
232 could all differ between environmental treatments experienced for that line in G1/G2 and P4.
233 The model contained fixed effects of the G1/G2 and P4 environmental treatments, pot order
234 effects, age effects, the effects of the maternal trait values for that line in G3 (difference from
235 the overall mean) and interactions of these (except for age×trait interactions and interactions

236 between traits). Note that we thus test whether strengths of maternal trait-based effects depend
237 on environmental conditions. Model selection was carried out as above. However, we
238 observed that selected models often had confidence intervals for the maternal effect slopes
239 that still overlapped with zero or with each other and we simplified such effects out of the
240 models. To interpret the results more easily and to have a graphical means to assess the
241 validity of mixed model predictions, we also fitted linear regressions to offspring trait –
242 maternal trait combinations. All statistical analyses were conducted using R (Team, 2005).

243

244 **Whole-genome bisulfite sequencing (WGBS)**

245 To investigate the impact of mild drought on genomic DNA methylation patterns, WGBS was
246 performed on pooled DNA extracted at day 29 after sowing from mature leaves of 12 Col
247 plants that were being subjected to control or water deficit treatments and on 10 day-old
248 seedlings derived from 5 independent C₁C₂ and S₁S₂ G₂ lines grown under standard *in vitro*
249 conditions. MethylC-seq library preparation and sequencing was performed by BGI
250 (Shenzhen, China) using standard Illumina protocols. Adapter and low-quality sequences
251 were trimmed using Trimming Galore v0.3.3. Mapping was performed on TAIR10 genome
252 annotation using Bismark v0.14.2 (Krueger & Andrews, 2011) and the parameters: --bowtie2,
253 -N 1, -p 3 (alignment); --ignore 5 --ignore_r2 5 --ignore_3prime_r2 1 (methylation extractor).
254 Only uniquely mapping reads were retained. The methylKit package v0.9.4 (Akalin *et al.*,
255 2012) was used to calculate differential methylation in individual positions (DMPs) or in 100
256 bp non-overlapping windows (DMRs). Significance of calculated differences was determined
257 using Fisher's exact test and Benjamin-Hochberg (BH) adjustment of *p*-values (FDR < 0.05)
258 and methylation difference cutoffs of 40% for CG, 20% for CHG and 20% for CHH.
259 Differentially methylated windows within 100bp of each other were merged to form larger
260 DMRs. Cytosine positions covered by more than 100 reads were not considered. For DMP
261 analysis only cytosines covered by a minimum of 6 (CG and CHG) and 10 (CHH) reads in all
262 libraries were considered. Bisulfite conversion rates were estimated by the number of
263 methylated cytosine calls in the chloroplast genome.

264

265 **RNA-seq**

266 To investigate the impact of mild drought on gene expression, we performed RNA-seq on
267 leaves isolated from stressed and non-stressed Col-0 plants. Leaf tissue was collected at 23
268 days after sowing from three Col-0 individuals grown on the Phenoscope under control and
269 mild drought conditions and from the same seed batch as the founding Col-0 individuals used

270 in the transgenerational design. Total RNA was extracted using the Qiagen RNAeasy
271 extraction kit and sequenced at the Genome Center of the Max Planck Institute for Plant
272 Breeding Research in Cologne, Germany. RNA-seq libraries were constructed using the
273 standard Illumina Truseq protocol and sequenced in an Illumina Hiseq 2500 machine.
274 Between 18.3 and 23.7 million reads were obtained per sample (average of 20.7) and aligned
275 to the TAIR10 reference genome using TopHat2 with default parameters (Kim *et al.*, 2013).
276 Reads aligning to multiple locations were removed using samtools' view with parameter -q 5
277 (Li *et al.*, 2009). After this filter, between 95.5 and 96.8 percent of the obtained reads were
278 aligned to the reference genome. The number of reads per transcript was counted using the
279 Bioconductor packages Rsamtools and ShortRead (Morgan *et al.*, 2009). Differential
280 expression between samples in control and drought conditions was calculated with the
281 DEseq2 package in R (Love *et al.*, 2014). Genes with q-values lower than 0.05 and log2FC
282 above 0.5 were considered as differentially expressed. TE differential expression was
283 analyzed using TETOOLS (Lerat *et al.*, 2016).

284

285 RNA-seq and MethylC-seq sequencing data have been deposited in the ENA short read
286 archive under project number PRJEB27682.

287

288 **Results**

289

290 **Mild drought induces immediate phenotypic plasticity**

291 Growth dynamics of the projected rosette area (PRA) in each generation where we imposed
292 mild drought indicated clear phenotypic plasticity in response to mild drought for the five
293 accessions analyzed, as expected (Tisne *et al.*, 2013). Indeed PRA decreased significantly
294 when accessions were grown under mild drought (generation G1, Fig. 2a) and reached values
295 that are on average 27% to 40% lower than in control conditions at an age of 29 days after
296 sowing, depending on the accession (G1, Fig. 2b).

297 We then compared, in as much detail as possible, phenotypic traits between the progeny of
298 plants whose parents experienced stress treatments for two consecutive generations and the
299 progeny of plants whose parents never experienced stress (Fig. 1; phenotyping at P4 compares
300 C₁C₂C₃ vs. S₁S₂C₃ trajectories). In both cases one final generation without a stress treatment
301 was included (C3), in order to detect only effects with some capacity to persist independently

302 of the presence of the environmental cue, and to remove direct effects of maternal
303 environments.

304 We did not find any effect of exposure to stress in the first two generations on initial and final
305 log(PRA) of the progeny of the third generation (P4). The finite mixture analysis of initial
306 log(PRA) indicated that the cohort is not composed of very different groups responding
307 differently to treatments. There are no hidden large heterogeneities among the plants installed
308 on the robot and for each accession, a single Gaussian component was always preferred. In
309 the case of Col, the preferred model did contain an effect of exposure to stress in G1/G2 on
310 average initial size in P4 ($p = 0.004$), with descendants of stressed plants having larger sizes
311 (log(PRA) difference 0.063, s.e. 0.022). We found no effects of exposure to stress in G1/G2
312 nor P4 on mean initial log(PRA) when taking line variation and pot order effects into account
313 in any of the accessions, hence the single significant effect in the mixture analysis should be
314 attributed to ignoring non-independence in the data. Unexpectedly, we found effects of stress
315 in P4 on initial log(PRA) heterogeneity (residual variance) of two accessions in that
316 generation (Supporting Information Table S1: Cvi and Bur). These effects must be spurious
317 (sampling effect), as the stress treatment has not started yet at this stage. Therefore, we do
318 model variance heterogeneity throughout but do not present nor interpret the results. For mean
319 final size, we find that all accessions except Sha show significant plasticity - their sizes are
320 larger in the control (Table 1).

321 Our gamm analysis indicated that relative growth rates (RGR) from days 13 to 16 and from
322 days 25 to 28 could be considered linear per individual (Figure 3, representative results for
323 accession Col). Notably, growth rate plasticity in response to drought is not permanent.
324 Indeed, relative growth rates near day 28 are very similar under control and stress conditions,
325 though obviously absolute plant size is smaller.

326 Modeling RGR demonstrates a steeper decrease from five to eight days after the water supply
327 is reduced in all accessions (Table 2, Fig. 3) and a corresponding decrease in the growth rate
328 intercept for the control group (a more steeply decreasing function has a more positive
329 intercept at day zero as a side effect). However, this initial decrease is followed by a recovery.
330 For three out of five accessions, plants subjected to mild drought acclimate and recover RGR
331 similar to that of control plants (days 25 to 28, Table 3, Fig. 3). For Cvi the recovery is
332 incomplete and for Sha we find some compensatory growth; RGR decreases less with age in
333 the stress group. There are no memory effects of exposure to stress in G1/G2 on average
334 growth rate values in P4. Different magnitudes of between-individual variance across the
335 trajectories do occur for Col, Cvi and Bur. The individual variation in relative growth rates is

336 larger from days 13 to 16 for the Col individuals that descend from parent that experienced
337 stress in G1/G2 ($p < 0.001$); for Bur the variance in the descendants of G1/G2 stress group is
338 smaller ($p = 0.022$). For Col, the growth rate variance from days 25 to 28 is smaller for the
339 descendants of the G1/G2 stress group ($p < 0.001$). For Cvi, this variance is larger in the
340 descendants of the G1/G2 stress group ($p < 0.001$). This pattern points out that amounts of
341 trait variation at the end of an experiment or at the moment where selection occurs could be
342 determined by intricate time-dependent variances in growth processes.

343

344 **Limited presence and persistence of maternal trait-based effects**

345 Models with maternal trait effects on individuals in P4 find in two out of 125 tests that the
346 slope of the maternal trait regression depends on the environmental regime experienced by
347 ancestors in G1/G2 (Supporting Information Table S2) and in two cases on the environmental
348 regime in P4. By inspecting the models per accession, we can make an assessment of whether
349 maternal trait slopes changed by mild stress would affect the persistence of these maternal
350 effects. This can be the case when slopes of traits on themselves have become larger in
351 absolute value or when a causal chain of traits has obtained a stronger weight (Kirkpatrick &
352 Lande, 1989). Only for compactness and in Col and Bur do we find a maternal trait-based
353 effect where the trait has an effect on itself. While for Col-0 the slope of the maternal effect is
354 changed in plants from lines that have experienced stress in previous generations, this is not
355 the case for Bur (Supporting Information Table S2). There are no enchainment maternal trait
356 effects that would lead to lagged responses over several generations.

357 When we inspect the maternal trait dependency (Fig. 4) of compactness in Col, we note that
358 the ranges of maternal trait values differ between descendants of stress and control treatments
359 in G1/G2. The results do show that maternal trait models have slopes that can depend on
360 historical and current environments and that the scope for a change in the persistence of
361 maternal effects due to mild stress is limited (Fig. 4). Indeed, the slopes of the effects are not
362 particularly strong, nor general across accessions and they do not seem a valid candidate for
363 persistent transmission of trait variation. Only 5 out of 125 tests for effects of maternal traits
364 on offspring were significant and had interpretable confidence intervals, which is very close
365 to the type I error rate. We therefore conclude that the number of heritable effects transmitted
366 through maternal trait-based effects is negligible.

367 In 13 out of 25 trait \times accession models, individual variation is enlarged in the stress
368 environment in P4 (Supporting Information Table S3), in 5 out of 25 models the variation

369 between individuals is larger among descendants of individuals stressed in G1/G2. In two
370 cases this variance is smaller.

371

372 **Mild drought induces DNA methylation variation and uncorrelated transcriptome** 373 **changes**

374 To complement the phenotypic analysis, we investigated the impact of mild drought on
375 genomic DNA methylation patterns using the reference accession Col-0, which shows the
376 strongest phenotypic response (Fig. 2b) and for which a wealth of epigenomic data are
377 available. WGBSeq was performed on DNA extracted at day 29 from leaves of pools of
378 treated and control plants at P4 (C1C2C3 descendants, Fig 1; Supporting Information Tables
379 S4 and S5). Overall, cytosine methylation levels are similar between control- and stress-
380 treated leaves (Supporting Information Fig. S1a), albeit slightly higher than in previous
381 reports (8.6% and 9% of methylated cytosines vs. 6.7%), presumably because of differences
382 in mapping and methylation calling methods as well as in the organs examined (Cokus *et al.*,
383 2008; Lister *et al.*, 2008). Other global measures, such as the distribution of methylation
384 between the three types of sites and annotations are also identical for control- and stress-
385 treated leaves (Supporting Information Fig. S1b and c). Thus, we conclude that mild drought
386 does not directly affect overall DNA methylation patterns in Arabidopsis.

387 To identify local differences, methylation levels were compared at individual cytosine
388 positions as well as in 100 bp windows for each of the three types of sites (CG, CHG and
389 CHH) separately (see Methods). Based on this approach, we could identify 286 differentially
390 methylated positions (DMPs) and 1360 differentially methylated regions (DMRs), most of
391 which are defined by single 100 bp windows (Supporting Information Tables S6 and S7). All
392 DMPs map to CG sites whereas most DMRs (95%) are CHH DMRs only (Fig. 5a). The vast
393 majority of CG DMPs (93%) are within methylated gene bodies (Fig. 5a and b) and they
394 reflect almost equally either increased or decreased methylation levels in treated plants
395 compared to controls, consistent with the notion that gene body methylation tends to vary
396 stochastically across generations at individual CG sites (Becker *et al.*, 2011; Schmitz *et al.*,
397 2011; Jiang *et al.*, 2014). On the other hand, CHH-DMRs are mainly located over TE
398 sequences and tend to reflect hypermethylation in treated plants (Fig. 5c and d, Supporting
399 Information Fig. S2).

400 As different TE families may show different sensitivity to environmental cues (Pecinka *et al.*,
401 2010; Yu *et al.*, 2013; Grandbastien, 2015; Matsunaga *et al.*, 2015; Quadrana *et al.*, 2016), we
402 assessed whether CHH-DMRs are preferentially localized over specific TE families. Out of

403 the 326 TE and other repeat families annotated in the TAIR10 Arabidopsis genome, 164 show
404 at least one DMR and 18 families are enriched in DMRs compared to the random expectation
405 (Fig. 5e). These include the LTR-retrotransposon family *ATCOPIA78*, which is known to be
406 sensitive to biotic and abiotic stress (Yu *et al.*, 2013; Quadrana *et al.*, 2016; Matsunaga *et al.*,
407 2015). On the other hand, only a small percentage of CHH DMRs caused by mild drought
408 overlap with DMRs that arose spontaneously in mutation accumulation lines (9.3%; Haggmann
409 *et al.*, 2015) or that were induced by hyperosmotic stress (1.9%; Wibowo *et al.*, 2016) (Fig.
410 5f). Thus, we conclude that mild drought induces a limited number of robust DNA
411 methylation changes over regions that are distinct from those subjected to stochastic or salt-
412 induced DNA methylation variation.

413 To investigate further the CHH-DMRs induced by mild drought, we compared their CHH
414 methylation level in different DNA methylation mutants (Stroud *et al.*, 2013) and found that
415 most correspond to regions targeted by the RNA-directed DNA methylation (RdDM)
416 pathway, which involves the DNA methyltransferase DRM2, rather than by the alternative
417 CHH maintenance methylation pathway mediated by the DNA methyltransferase CMT2 (Fig.
418 5g). Consistent with these findings, TE sequences overlapping drought induced CHH-DMRs
419 have a high abundance of matching 24nt small RNAs (Fig. 5h). Moreover, no correlation was
420 detected between drought induced CHH-DMRs and regions subjected to active DNA
421 demethylation (*rdd* mutant; Fig.5g). In conclusion, mild drought directly affects mainly
422 sequences targeted by RdDM.

423 To determine if genes with changes in DNA methylation near or within them are drought
424 stress responsive, we performed RNA-seq on leaves isolated from Col-0 plants directly
425 exposed to mild drought or control treatments and grown for 3 weeks on the Phenoscope.
426 Consistent with the results of previous studies of the transcriptional response to mild drought
427 stress (Cubillos *et al.*, 2014; Clauw *et al.*, 2015), differential analysis of the two RNA-seq
428 datasets identified significant changes in steady state mRNA levels for 468 genes (FDR <
429 0.05, >0.5 log₂ fold change, 205 and 263 genes with lower and higher expression under mild
430 drought, respectively; Supporting Information Table S8), but not for any of the annotated TE
431 sequences. Gene Ontology analysis revealed enrichment for several stress-related categories,
432 including response to water deprivation, for which the highest significance level was
433 observed. However, none of the genes known to be involved in DNA (de)methylation
434 appeared to be affected by mild drought, which leaves the question open as to which factors
435 induce DNA methylation changes during mild drought.

436 Among the 468 genes detected as transcriptionally responsive to mild drought in our
437 conditions, only two are affected by CG DMPs, which are likely inconsequential given the
438 lack of function associated with gene body methylation. Another two are located less than
439 500bp from a DMR (Supporting Information Fig. S3a). These two DMRs are of the CHH
440 type but do not correspond to annotated TE sequences. One DMR maps to the promoter
441 region of gene *AT5G35735*, which encodes an auxin responsive protein of unknown function.
442 The other DMR is located within the first intron of gene *AT3G10340*, which encodes a
443 putative phenylalanine ammonia-lyase that may be involved in plant defense against biotic and
444 abiotic stresses (Raes *et al.*, 2003). Given the first intron large size (1.3kb), it likely contains
445 regulatory sequences (Morello & Breviario, 2008). Moreover, hypermethylation of the
446 promoter DMR of *AT5G35735* and hypomethylation of the intronic DMR of *AT3G10340* in
447 response to mild drought are associated with down and up regulation, respectively
448 (Supporting Information Fig. S3b). Taken together, these findings suggest a causal link
449 between altered DNA methylation and altered gene expression for these two genes. However,
450 the observation that most genes affected by mild drought are not proximal to drought-induced
451 DMPs or DMRs indicates that changes in DNA methylation have a marginal role in the
452 phenotypic response of plants to mild drought.

453

454 **DNA methylation variation is not transmitted to the progeny**

455 Finally, we tested whether the DNA methylation changes directly induced by mild drought
456 could be transmitted from stressed plants to their progeny. Taking into consideration the
457 possibility of cumulative effects over successive generations of growth under stress, G3
458 progenies of Col-0 C₁C₂ and S₁S₂ plants were chosen for further analysis (Fig. 1a). WGBseq
459 was performed on DNA extracted from unstressed individuals of the progeny of five Col C₁C₂
460 and S₁S₂ founder lines (Methods and Supporting Information Tables S4 and S5). Differential
461 DNA methylation was investigated as described above using the five C₁C₂ or S₁S₂ progenies
462 as biological replicates. Following this approach, no single consistent DMR could be
463 identified between the two types of progeny. However, there was a marginal increase in the
464 amount of stochastic variation in DNA methylation for the three sequence contexts among
465 progenies derived from the five stressed parental lines (Supporting Information Fig. S4). In
466 conclusion, our results suggest that targeted and specific DNA methylation changes induced
467 by mild drought are not transmitted to the next generation, although stress exposure may
468 increase methylome heterogeneity among progeny of stressed plants.

469

470 **Discussion**

471

472 It has been proposed that exposure to environmental cues can trigger phenotypic changes that
473 are inherited for more than one generation, and that this occurs through epigenetic
474 mechanisms (Bossdorf *et al.*, 2008; Richards *et al.*, 2017). In this study, we showed that water
475 deficit applied before the reproductive stage in two successive generations affects the
476 vegetative growth of individuals negatively. Memory effects of mild drought stress after two
477 successive generations of exposure are limited to changes in amounts of individual variation
478 in phenotypic traits, but the variance can be increased or decreased depending on the
479 accession and trait considered. Furthermore, although changes in DNA methylation in
480 response to mild drought were observed, these were only marginally associated with changes
481 in gene expression and were not transmitted to the immediate progeny of the affected
482 individuals, even after two successive generations of exposure to stress. In other words, there
483 is no substantial evidence that drought stress would modify the heritability of methylation
484 patterns and of maternal trait-based effects. Therefore, our results add to the growing body of
485 evidence against transgenerational epigenetic variation being a common response of plants to
486 changes in the environment.

487

488 **Intergenerational plasticity is limited and does not lead to transgenerational effects**

489 Stresses consistently affect the expression of a large number of genes and induce in a number
490 of cases CHH hyper or hypomethylation of a variable number of TE sequences and other
491 repeat sequences (for example Downen *et al.*, 2012; Eichten & Springer, 2015; Secco *et al.*,
492 2015; Wibowo *et al.*, 2016; this study). However, gene expression changes rarely correlate
493 with DNA methylation changes (Meng *et al.*, 2016) and the extent of the latter as well as the
494 mechanisms at play may differ radically between plant species for a given stress (Secco *et al.*,
495 2015). Furthermore, DNA methylation changes may or may not be transmitted to the next
496 generation, for reasons that are unclear. Thus, while in *Arabidopsis* many of the CHH-DMRs
497 induced by salt stress are transmitted to the immediate progeny (Wibowo *et al.*, 2016), this is
498 not the case for the CHH-DMRs induced by mild drought (this study) and in rice there was
499 also no transmission of the CHH-DMRs induced by phosphate starvation (Secco *et al.*, 2015).
500 Thus, evidence so far points to a clear effect of environmental factors in triggering DNA
501 methylation changes, which however do not persist across more than one generation. Indeed,
502 different mechanisms preventing transgenerational transmission of environmentally induced

503 epigenetic states have been described (Baubec *et al.*, 2014; Crevillen *et al.*, 2014; Iwasaki &
504 Paszkowski, 2014). Nonetheless, true transgenerational epigenetic variation exists in nature
505 (Quadrana & Colot, 2016) and what generates it remains unresolved. Analyses of natural
506 populations are now just beginning to investigate this question, with no clear answer so far,
507 except that most DNA methylation variants seen in nature are likely caused by DNA sequence
508 variation and therefore are by definition not truly epigenetic (Schmitz *et al.*, 2013; Li *et al.*,
509 2014; Dubin *et al.*, 2015; Kawakatsu *et al.*, 2016b; Niederhuth *et al.*, 2016; Quadrana *et al.*,
510 2016).

511

512 At the morphological level, we detected different responses between accessions, with both
513 plasticity and some trait-based maternal effects playing a role. Maternal trait-based effects
514 with potentially lasting effects occur but for rosette compactness only. Such effects should
515 dampen quickly when selection does not favor increased compactness for example and these
516 maternal effects are potentially swamped by environmental variability. Drought stress
517 changes trait variances but not slopes of maternal trait-based effects. No new significant
518 slopes were created. Therefore mild drought stress did not change this presumed mechanism
519 of non-genetic heritability.

520

521

522 **Plasticity is likely adaptive, a memory effect not**

523 Transgenerational effects are often presumed to be adaptive. Models show that
524 environmentally induced epiallelic variation can be favored over purely stochastic switching
525 (Furrow & Feldman, 2014). The general lack of such effects in our experiment might indicate
526 that they are actually unnecessary in the range of imposed environments. The remaining
527 responses observed would then rather be of a non-adaptive nature and reflect mechanistic
528 constraints in plant development. In agreement with models of adaptation (Kuijper & Hoyle,
529 2015), we find that responses by means of phenotypic plasticity are stronger than by maternal
530 effects. The changes in trait variance could indicate that mild drought stress rather affects the
531 predictability of the near future, without much of a consistent trend in expectations. Our
532 observed changes might then be more in support of a bet-hedging strategy (e.g., Crean &
533 Marshall, 2009).

534 Modeling suggests a potential for DNA methylation-based transgenerational epigenetics to
535 endow plants with a means to generate adaptive heritable phenotypic variation in response to
536 changing environments (Bossdorf *et al.*, 2008; Geoghegan & Spencer, 2013b; Geoghegan &

537 Spencer, 2013a; Uller *et al.*, 2015; Kronholm & Collins, 2016). We did not detect this. The
538 absence of a clear change in maternal-trait based effects seems to indicate that mild drought
539 stress does not induce strong physiological changes that would affect transmission of
540 information or resources. Similarly, we observed that relative growth rates respond to this
541 stress in a transient manner, clearly affecting final sizes but with return to control-like relative
542 growth rates after stress treatment. It has been proposed to see phenotypes as weighted sums
543 of ancestral contributions (Sultan, 2017). Similarly to Tallis' vision of ancestral genotypic
544 regression (Tallis, 1987), phenotypic plasticity would be broadened to include effects of
545 ancestral environments. Maternal trait-based effects are such ancestral genotypic \times
546 environmental regressions. We find that contributions of ancestral states were limited and
547 decay quickly.

548

549 In conclusion, our study provides strong support to the notion that plants first respond to
550 physiological stresses through well-defined and conserved transcriptional networks (Juenger,
551 2013) (Ding *et al.*, 2014; Clauw *et al.*, 2015) or immediate parental influences on offspring
552 phenotypes (Herman & Sultan, 2011; Wibowo *et al.*, 2016). Whether transgenerational
553 epigenetic variation in nature is caused by more dramatic environmental conditions than those
554 tested so far in the laboratory, or by combinations of several mild stresses, or by mutations in
555 genes, such as *Arabidopsis DDMI*, that are involved in the epigenetic control of TE remains
556 to be determined (Quadrana & Colot, 2016).

557

558 **Acknowledgments**

559 We thank members of the Colot lab for discussions. This work was supported by funding
560 from the Agence Nationale de la Recherche (project MEMOSTRESS, grant n° ANR-12-
561 ADAP-0020-01 (to V.C, O.L. and T.J.M.V.D.) and the European Commission Framework
562 Programme 7, ERC Starting Grant 'DECODE' / ERC-2009-StG-243359 (to O.L). The IJPB
563 benefits from the support of the LabEx Saclay Plant Sciences-SPS (ANR-10-LABX-0040-
564 SPS). ABS was supported by a postdoctoral fellowship from the Brazilian National Council
565 for Scientific and Technological Development (CNPq – Brazil). LQ was recipient of
566 postdoctoral fellowships from the ANR-10-LABX-54 MEMOLIFE and ANR-11-IDEX-0001-
567 02 PSL Research University.

568

569 **Author contributions**

570 T.J.M.V.D., A.B.S., O.L., and V.C. planned and designed research; A.B.S., E.G., A.M., L.B.
571 and S.T. performed experiments and collected the data; T.J.M.V.D., A.B.S., L.Q., analyzed
572 the data; J.J.G. contributed unpublished results; T.J.M.V.D., A.B.S., O.L. and V.C. wrote the
573 manuscript with the help of all authors.

574

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779
780

781 **Table 1.** Effects of environmental states in G1/G2 and P4 on the mean final projected rosette area
 782 log(PRA) in generation P4. Confidence interval results from linear mixed models per accession, based
 783 on parameter estimates of minimum adequate models are shown. Symbol "*" indicates accessions
 784 where the variance between lines is retained in the model. LRT = Likelihood Ratio Test.

Accession	Memory of stress in G1/G2	LRT	Plasticity P4 (Control - Stress)	LRT
COL	-	NS	[0.197 - 0.375]	p < 0.001
BUR	-	NS	[0.317 - 0.546]	p < 0.001
CVI*	-	NS	[0.394 - 0.557]	p < 0.001
TSU	-	NS	[0.250 - 0.384]	p < 0.001
SHA	-	NS		NS

785

786 **Table 2.** Effects of environmental states in generations G1/G2 and P4 on the mean relative growth rate
 787 (RGR) from days 13 to 16 in P4. Results from linear mixed model analysis and model after selection.
 788 In the table, accessions with random effects that differ in line/individual variance between the two
 789 G1/G2 treatment levels are indicated by symbol "***". Symbol "*" indicates accessions where the
 790 variance between lines is retained in the model. "#" indicates accessions for which the pot effects
 791 (linear effect of pot number on the robot) were retained.

Accession	Memory effect	LRT test	Plasticity (Intercept difference between Control - Stress groups)	plasticity x Day slopes	LRT test
COL**		NS	-0.186	Stress -0.046 Control - Stress 0.039	p < 0.001
BUR***#		NS	[-0.32, -0.21]	Stress [-0.073, -0.060] Control-Stress [0.045, 0.060]	p < 0.001
CVI		NS	[-0.22, -0.03]	Stress [-0.058, -0.037] Control - Stress [0.017, 0.046]	p < 0.001
TSU*		NS	[-0.25, -0.15]	Stress [-0.062, -0.049] Control - Stress [0.033, 0.049]	p < 0.001
SHA*		NS	[-0.59, -0.39]	Stress [-0.107, -0.080] Control - Stress [0.077, 0.106]	p < 0.001

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793

794 **Table 3.** Effects of environmental states in G1/G2 and P4 on the mean relative growth rate from days
 795 25 to 28 in P4. Results from linear mixed model analysis. In the table, accessions with random effects
 796 that differ in line/individual variance between G1/G2 groups are indicated by "***". Symbol "*" indicates
 797 accessions where the variance between lines is retained in the model. "#" indicates
 798 accessions for which the pot effects (linear effect of pot number on the robot) were retained.

Accession	Memory effect	LRT test	Plasticity (Intercept difference between Control - Stress groups)	plasticity x Day slopes	LRT test
COL***#		NS			NS
BUR*		NS			NS
CVI**		NS	-0.206	Stress -0.017 Control - Stress 0.011	p < 0.001
TSU*#		NS			NS
SHA		NS	[0.00, 0.24]	Stress [-0.003, -0.002] Control - Stress [-0.014, -0.001]	p = 0.021

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802

803 **Figure legends**

804

805 **Fig. 1.** Schematic representation of the multigenerational experimental design. G, generation
806 of growth; P, phenotyping experiment.

807

808 **Fig. 2.** Descriptive analysis of growth curves for projected rosette area (PRA) in the first
809 generation (G1) for the five accessions grown under control or drought (stress) conditions.
810 Individuals that remained below 1 cm² PRA by the end of the experiment or that died
811 prematurely are not shown.

812 (a) Kinetics of shoot size estimated by daily measurements of PRA. Growth curves of plants
813 in control conditions are in blue, those that experienced mild drought (stress) in red. Black
814 lines represent the averages per group. (b) Box and whiskers plot showing PRA on day 29 for
815 the plants in each accession × treatment combination. Inset photographs show representative
816 plants per accession × treatment.

817

818 **Fig. 3.** The time-dependent pattern of relative growth rates as predicted for the first pot on the
819 robot (the model accounts for pot order) and for accession Col-0 based on generalized
820 additive mixed models (gamms). On the x-axis the age of the plants in days is given. Plants
821 are nine days old after sowing when the recording starts. Four panels show the raw data per
822 treatment combination. Above and to the right of each column and row, predicted trajectories
823 are grouped for two panels each time so that pairwise comparisons between G1/G2 (Memory)
824 and P4 (Plasticity) treatment levels can be made. The raw data values are shown in grey
825 together with a predicted relative growth rate trajectory per combination of treatments in
826 G1/G2 (Memory) and P4 (Plasticity). P4 treatments are shown in blue (Control) or black
827 (Stress). The graphs demonstrate clear growth plasticity in response to mild drought and that
828 plants manage to compensate the initial drop in relative growth rate shortly after the mild
829 drought stress has reached a stable level at day 20. Note that there are very few plants with
830 outlying patterns and that these have very low growth rates for a restricted age window only.

831

832 **Fig. 4.** Effects of maternal traits in Col-0. Dependencies of offspring trait values on maternal
833 trait values are shown for log-transformed rosette compactness in dependence on two
834 maternal trait values. In the mixed model analysis, we detected significant effects of maternal
835 compactness but not of maternal Mean Red value. Linear regressions are estimated per

836 ancestral environment. Data points are red for individuals with ancestors under stress in
837 G1/G2, black for individuals with ancestors under control.

838

839 **Fig. 5.** Characterization of stress-induced local DNA methylation changes.

840 (a) Number of DMPs and DMRs for each sequence context (CG, CHG and CHH). (b)
841 Annotation of DMPs and DMRs in relation to genes, TEs and intergenic regions. (c)
842 Distribution of local gains and losses of DNA methylation across DMPs and DMRs. (d)
843 Example of CHH DMRs on a TE. (e) Graphical representation of the 18 TE families that
844 show more DMRs than expected by random (p -value < 0.01). (f) Overlap (including 500bp
845 flanking windows) of DMRs induced by mild drought and DMRs found in mutation
846 accumulation (MA) lines (Becker *et al.*, 2011; Schmitz *et al.*, 2011) or induced by
847 hyperosmotic stress (Wibowo *et al.*, 2016) (g) Hierarchical clustering based on average CHH
848 methylation levels in wild-type (wt) and mutants for the RdDM (*rdr2*, *ago4* and *drd1*), CMT2
849 (*ddm1* and *cmt2*) and DNA demethylation (*rdd*) pathways in regions overlapping hyper or
850 hypermethylated CHH-DMRs. (h) Abundance of 24nt siRNAs in random or CHH-DMR
851 containing TEs.

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854

855 **Supporting Information legends**

856

857 **Fig. S1.** Genome-wide DNA methylation patterns are similar between leaves of stressed and
858 non-stressed plants.

859 **Fig. S2.** Chromosomal distribution of local gains and losses of DNA methylation found
860 between leaves of stressed and non-stressed plants.

861 **Fig. S3.** Differential methylation is weakly correlated to changes in gene expression in
862 response to mild drought.

863 **Fig. S4.** The progenies of stressed lines exhibit increased methylome instability.

864

865

866 **Table S1.** Effects of environmental state in G1 and G4 on the residual variance in initial
867 log(PRA), projected rosette area.

868 **Table S2.** Estimates of trait-based maternal effects per accession.

869 **Table S3.** Estimates of Individual within-line variances in models with trait-based maternal
870 effects per accession.

871 **Table S4.** Summary statistics of whole genome bisulfite sequencing data.

872 **Table S5.** Total fraction of methycytosines and distribution in each sequence context

873 **Table S6.** List of differentially methylated positions.

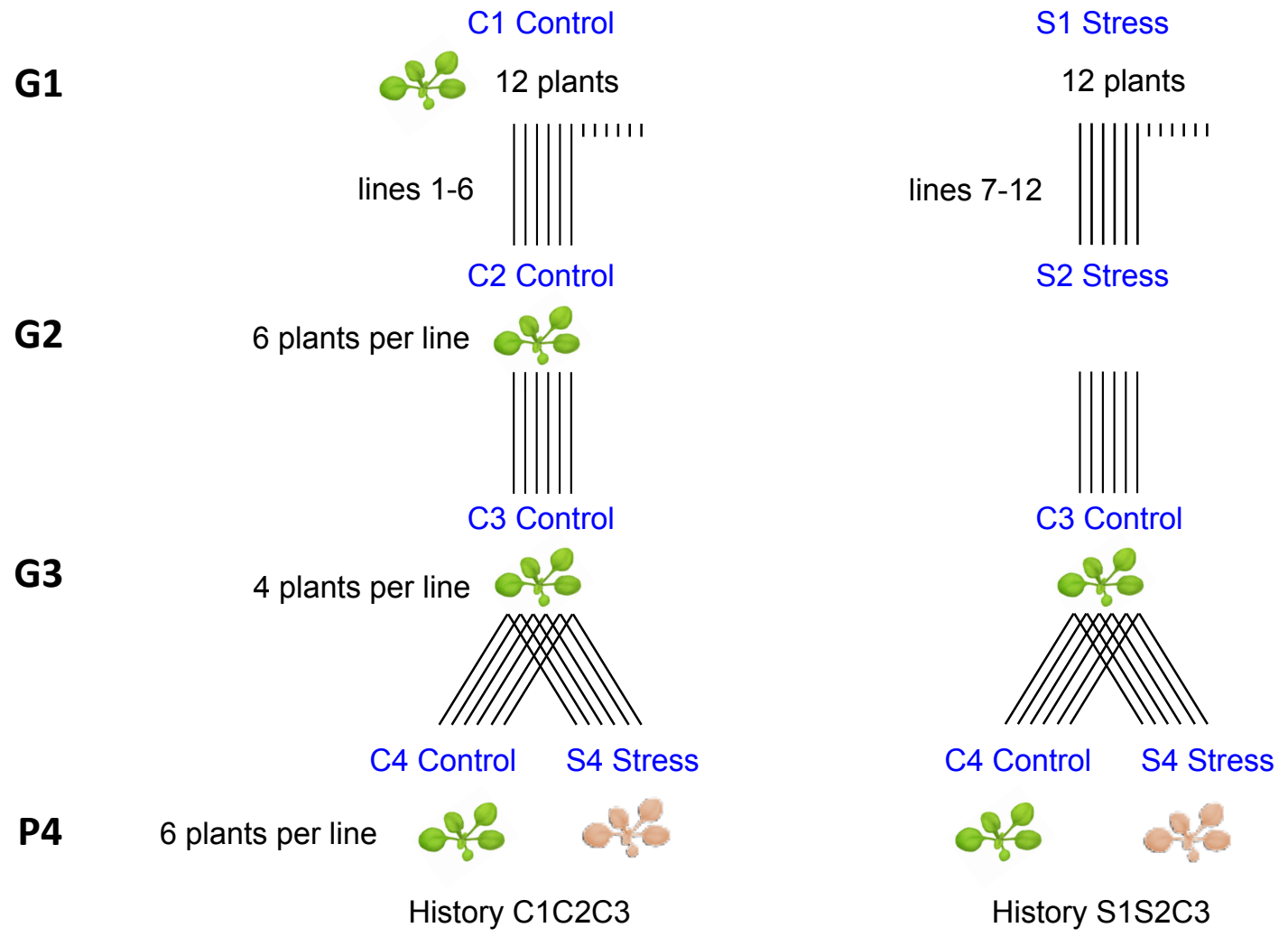
874 **Table S7.** List of differentially methylated regions.

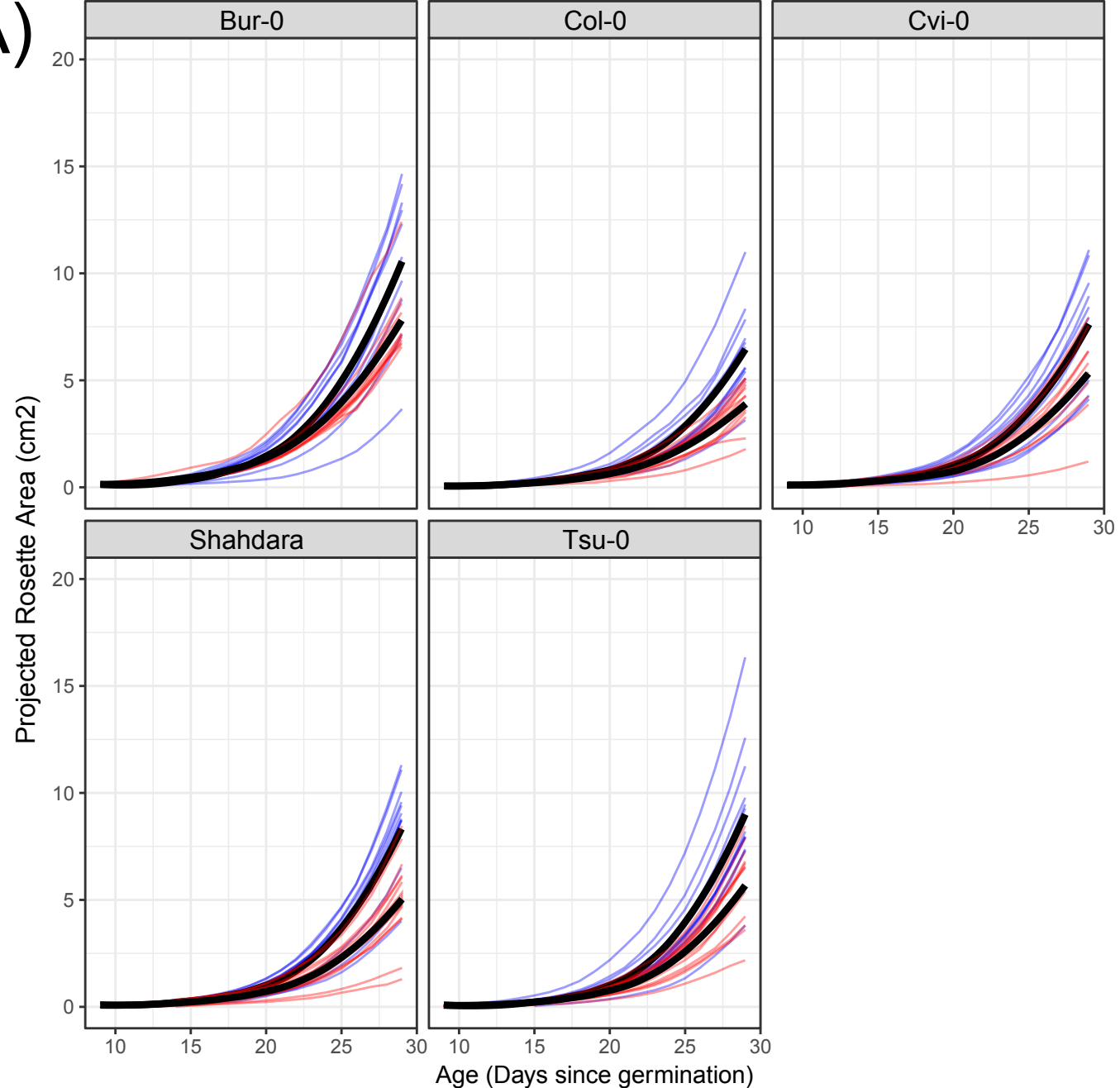
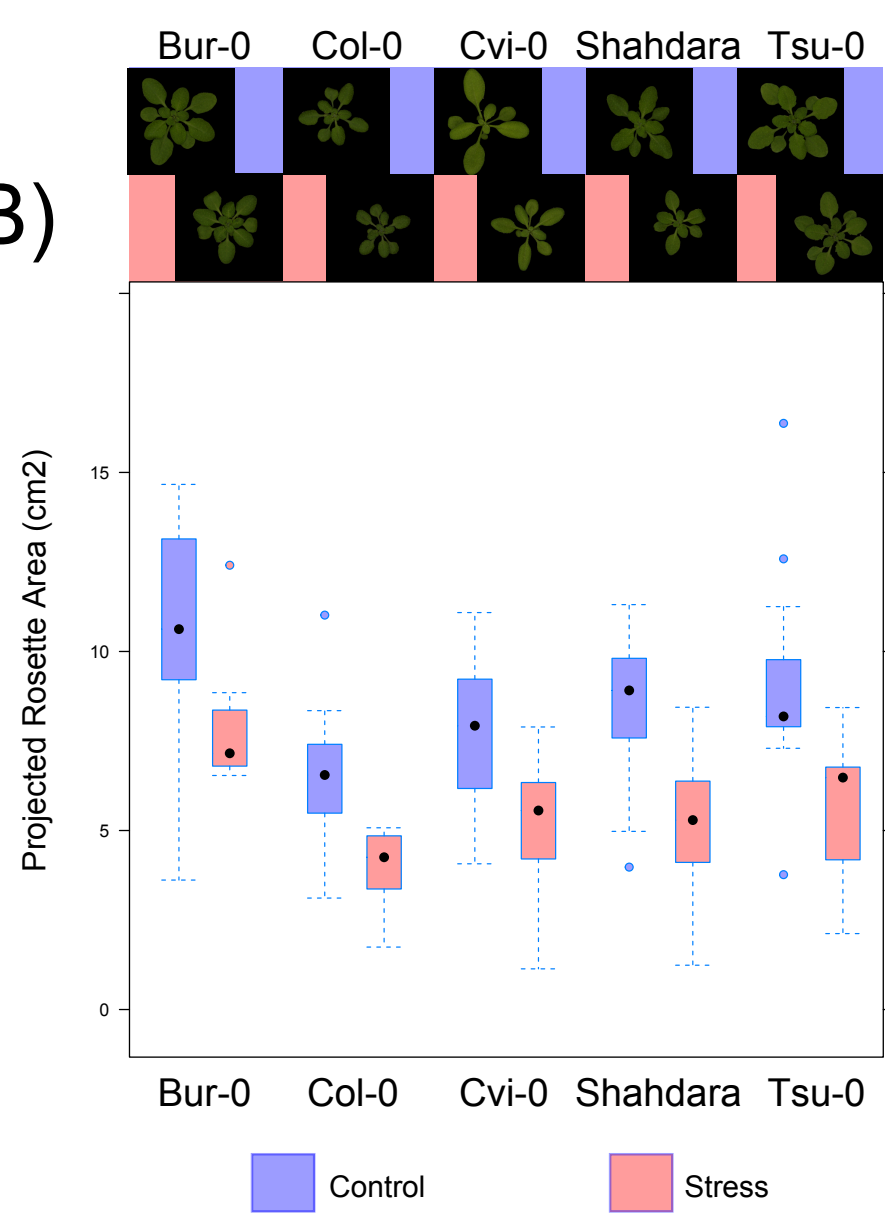
875 **Table S8.** List of differentially expressed genes.

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878



(A)**(B)**

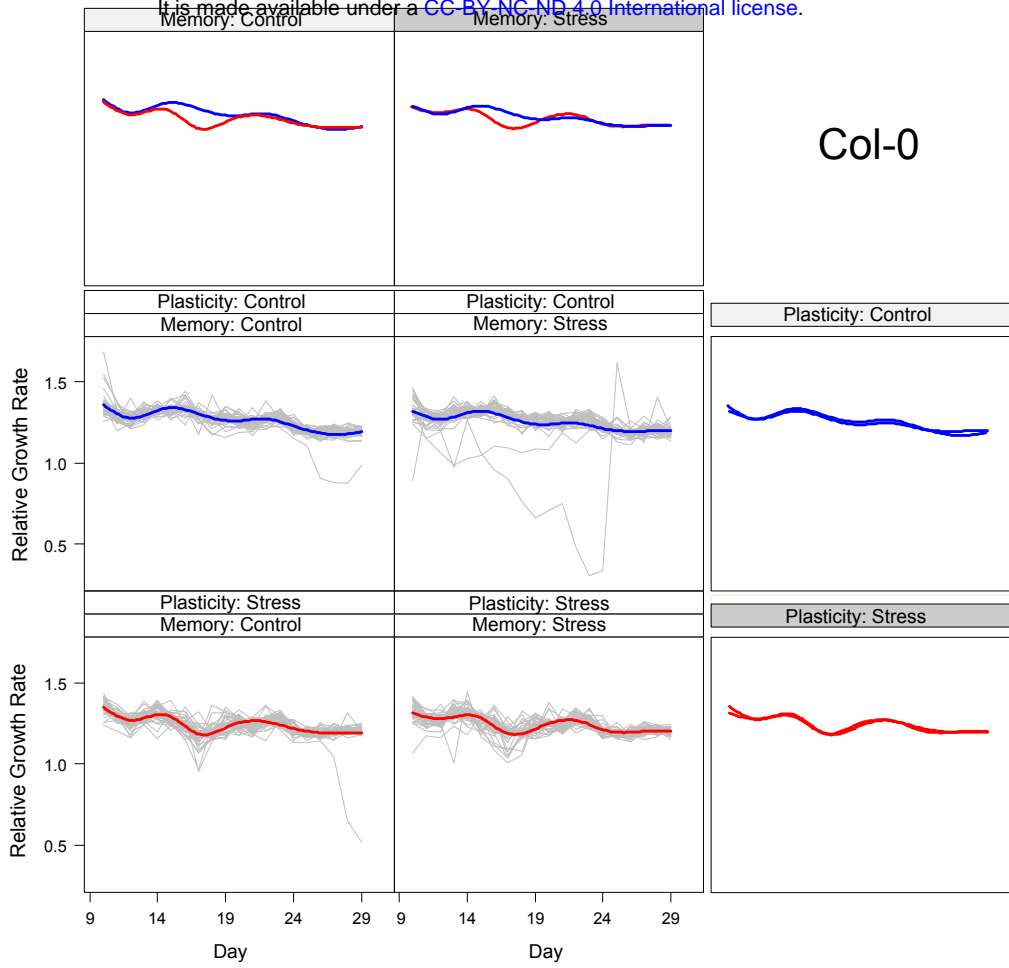


Figure 4

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